THE BIOLOGY OF ASCIDIA NIGRA (SAVIGNY). II. THE DEVELOPMENT AND SURVIVAL OF YOUNG ASCIDIANS [1]

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In the first paper in this series (Goodbody, 1962) a description was given of the survival and mortality of ascidians after they first appeared as black animals visible to the naked eye. Because of the technique used for studying those populations it was believed that most of them were about four weeks old when first recorded, and no information was available on the survival of animals of younger age. The work outlined in the present paper was designed to study two things: first, to study the development of ascidians from fertilization of the egg to the appearance of black pigment in the functional animal, with a view to producing a time scale for the different phases of development. Second, to study the survival of young animals from first settlement to the appearance of black pigment, and to determine whether there was any relationship between mortality rate and the different stages of development. While this has been achieved in some measure, it will become apparent that a far more fundamental problem emerges from it which will require further study. This is the relation of changes in the biotic environment to patterns of survival.

METHODS

Populations of young ascidians were obtained either artificially in the laboratory or through natural settlement on glass slides in the sea. Laboratory-reared populations were obtained through artificial fertilization of ascidian eggs: these hatched in 10 hours and the larvae were then pipetted gently into a perspex (lucite) settling box. This box, approximately 4 inches square and $2\frac{1}{2}$ inches in height, has walls of black perspex, a base of clear transparent perspex and an open top. The box is designed so that four standard microscope slides can be fitted, standing up edge to edge along each wall, in such a way that the inside of the box is lined with glass. One face of each slide is gently ground with carborundum to provide a roughened surface for settlement; by means of small clips at the top the slides can be secured with this roughened surface facing the inside of the box. With the slides in place and the box filled with water, the larvae were pipetted into it and the box then placed on a stand in an aquarium tank so that the flow of water outside the box kept the water inside cool. The clear base to the box allows light to come into it from below as well as from above: the larvae, which tend to settle on dark surfaces, were thus induced to settle on the glass slides backed by the dark wall of the box. The box was left thus for about six hours until such time as the larvae had settled.

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Attached larvae can be seen readily with the naked eye when a slide is withdrawn from the water.

When settlement was complete the slides were transferred to an open rack in the aquarium and left there overnight to ensure proper attachment before they were further disturbed. The following day each slide was transferred in turn to a perspex dish mounted on the moving stage of a binocular microscope. This dish is designed to contain sufficient water to cover the slides adequately during inspection, and has two projecting ledges on the inside base to enable each slide to be positioned in exactly the same place at successive inspections. In this way, and utilizing the graduated scales of the moving stage, it was possible to plot accurately the position of every young ascidian on each slide. When this was complete the slides were mounted edge to edge in a perspex frame backed by black perspex. (For further details of these frames, see Goodbody, 1961.) The black backing to the slides in the frames served two functions: it prevented any settlement of other organisms on the back of the slides which hence continued to fit properly into the microscope dish, and by providing a dark background it encouraged settlement of other organisms on the surface occupied by the ascidians, thus providing a natural community in which the ascidians could develop. These frames containing the slides were then suspended in the sea from floating rafts as described earlier (Goodbody, 1962). At intervals throughout the following six weeks the frames were carefully removed to the laboratory in a bucket of water; the slides were removed to a rack in the aquarium and examined in turn to determine how many of the original population still survived. To avoid any possible contamination while in the laboratory, special containers were reserved and used only for the transport and handling of these frames.

Naturally-occurring populations were also obtained on microscope slides. Clean slides similar to those described above were fitted into frames and placed in the sea, suspended from a raft. At intervals the slides were removed and inspected for newly settled larvae of *Ascidia nigra,* which can be identified at this stage (*cf.* Goodbody, 1961). Although newly settled larvae were repeatedly observed in small numbers, only two populations of sufficient size, and each comprising a natural cohort of animals derived from a single settlement, have been recorded and followed through their subsequent history. These two, however, provide a valuable supplement to the data obtained from the artificially settled populations.

The data on the developmental stages of the young ascidian were first worked out at Bermuda and have been confirmed and added to from observation and measurement of animals within these populations. Measurements were made with the aid of a micrometer eye-piece. All the data on survival were obtained at Port Royal, Jamaica.

The Sequence of Early Development

The sequence of events leading from the fertilization of the egg to the appearance of a black ascidian, visible to the naked eye, can be divided into stages as follows: embryonic development, the free swimming larva, settlement and metamorphosis, the functional protoascidian with two protostigmata on each side, the six protostigmata stage, twelve stigmata, six rows of eight stigmata, twelve rows of

stigmata and developing red pigmentation, and finally the opaque black ascidian. This whole sequence of events takes 19 days from the fertilization of the egg, at temperatures in the vicinity of 27° C. Illustrations of comparable stages in young ascidians are to be found in the papers of Berrill (1935, 1947).

The unfertilized egg measures about 160 μ in diameter; fertilization is external and the first cleavage (under laboratory conditions) follows in about 30 minutes. Subsequent cleavages follow in rapid succession, and the free swimming larva hatches 9 to 10 hours after fertilization. Grave (1935) gives a developmental time of 8 hours for this species at temperatures between 27 and 29° C. The same author's (1925) developmental time of 6 hours and 38 minutes was recorded at the exceptionally high temperature of 33° C. The total length of the larva is about 825 μ, of which 200 μ comprise the length of the body and the remaining 625 μ the tail. Larval behavior has not been studied in detail but, in common with most other ascidian species, the larvae of *A. nigra* are at first positively, and later negatively, phototropic. They are probably actively attracted towards iron, as submerged iron structures, which are not painted, often have dense growths of this species attached to them. Grave and Nicoll (1940) showed that iron accelerates metamorphosis in *A. nigra*.

Settlement of larvae under laboratory conditions usually occurs within 3 to 6 hours of hatching but has sometimes been delayed for as long as 12 hours. Grave and Nicoll (1940) give times ranging from $7\frac{1}{2}$ to 30 hours at Tortugas, Florida, according to the time of year. In field conditions an extended larval life may be more common, particularly if the larvae take a long time to find a suitable substratum on which to settle. When settlement is complete the tissues of the tail, except for the surrounding test, are resorbed into the body of the animal and accumulate there as a ball of nutrient reserves which nourish the growing animal until the gut is functional; these reserves have usually completely disappeared within 24 hours of the commencement of feeding by the functional animal. In the first stages of metamorphosis, when the tail has just been resorbed, the animal measures about 200 μ in length but this increases to 250 μ by the time the functional protoascidian commences feeding; this stage is reached about 45 hours after settlement of the larva.

The sequence of events from settlement of the larva is summarized in Table I, but it must be emphasized that there is some variation in the times and sizes given. A new stage of differentiation will only occur when the animal has reached a definite size: under conditions of poor food supply, growth may be retarded and hence the time scale will be correspondingly lengthened.

The functional protoascidian has two protostigmata on each side of the branchial sac, a single branchial aperture, paired peribranchial apertures, and a functional gut with oesophagus, stomach and intestine. Simultaneously with the commencement of feeding the first renal vesicle can be seen, close to the oesophagus. During the subsequent two days there is little new differentiation but the animal grows from about 250 μ to 550 μ and at the same time the nutrient reserves from the tail tissues completely disappears. Between the fifth and ninth days, and while the animal continues to grow, the original two protostigmata divide in such a way that there is formed, first a single row of six protostigmata, and then six rows of definitive stigmata on each side. There is considerable variation in the sequence

Time scale for the development of young Ascidia nigra from settlement to the appearance of black pigment

Time from settlement of larva	State of development	Approximate length (μ)
24 hours	Branchial siphon, 2 protostigmata, gut and heart just visible. Heart just commencing to beat. Large food reserves.	200
45–48 hours	Functional protoascidian with 2 pairs of protostigmata, food in gut, 1 renal vesicle. Food reserve small. Paired peribranchial apertures.	200–300
48–96 hours	Growth of the protoascidian and complete disappearance of food reserve. No new differentiation.	250–625
5–6 days	Differentiation of 6 protostigmata on each side. Branchial siphon develops lobes and red pigment spots between them. At least three renal vesicles.	500–700
7–8 days	Six protostigmata divide into 6 pairs of stigmata. Peribranchial apertures move toward mid-line and fuse. Four branchial tentacles visible. At least 9 renal vesicles.	700–1150
8–12 days	Six rows of stigmata completed. Peribranchial siphon becomes lobulated and develops red pigment spots.	1125–1875
13–15 days	Twelve rows of stigmata completed. Red pigmentation begins to develop.	1800–2400
19 days	Opaque black all over.	2750

of events occurring, and sometimes the protostigmata in the center of the row of six will have divided into three or more parts before the outer ones have completed their first division. On the fifth or sixth day the branchial siphon becomes lobed on its margins and red pigment spots develop between the adjacent lobes. The two peribranchial apertures begin to move towards the dorsal mid-line on about the seventh day, and by the eighth they have fused to form a single atrial siphon. Grave (1925) recorded fusion of the peribranchial apertures between the seventh and ninth days.

After the ninth day there appears to be a slight pause as further growth occurs up to a maximum of nearly 1900 μ, before the six rows of stigmata again divide to give twelve rows of stigmata on either side. It is at this stage, between the thirteenth and fifteenth days for normally growing animals, that the first signs of pigmentation appear. The pigment arises as a deep red coloration all over the animal which gets progressively darker until the eighteenth day from settlement, and at a size of about 2750 μ, the animal is so black that no further details of internal development can be observed.

Developmental times for other species of simple ascidian have been given by Berrill (1935) and Millar (1951, 1954). For *Ascidiella aspersa* (Müller) and

Ciona intestinalis (Linnaeus) at 16° C., Berrill records hatching after 24 hours; commencement of heart beat at 150 hours; appearance of functional protostigmata at 260 hours, and six rows of eight stigmata at 6 to 8 weeks. In comparison with these data for *Ciona* and *Ascidiella,* and allowing for a difference of 11° C. in the environmental temperature, the development of *A. nigra* seems extremely rapid. All the figures given are consistent with its development occurring six times faster than that of the other two species. Millar records the functional protostigmata stage as being reached in 10 days in both *Pyura squamulosa* (Alder) and *P. microcosmus* (Savigny) at temperatures in the vicinity of 20° C., which again is about six times as long as *A. nigra* takes to reach the same stage at 27° C.

Survival

As indicated earlier, survival has been studied in two types of poulation. In the first place there are data for seven different populations derived from artificial fertilization and subsequent settlement in the laboratory. As soon as settlement was complete and metamorphosis had begun, these populations were transferred to frames in the sea. Second, there are data from two other populations which settled naturally on microscope slides placed in frames in the sea for that purpose. The environmental histories of these different types of population are different, and comparison of the two must be made with caution. The populations derived from artificial fertilizations were allowed to settle on completely clean slides on which there were no competitors at the times of settlement; they were, in fact, the first colonizers of these slides and other colonizers apeared only after the slides had been placed in the sea, usually about 12 hours after settlement had taken place. This is an unnatural situation. The other populations appeared on the slides only when a sessile community had already begun to develop: in one of them, Group H, the ascidian larvae did not settle in the community until it was 16 days old; in the other (I) they settled after the slides had been in the sea for only two days.

The data on these nine populations are presented in Tables II and III. Table II shows the total number of animals in each population, together with the date of settlement and depth at which it was reared. In Table III the survival of each population is presented in terms of initial cohorts of 1000 animals each. Special note should be taken of population H, which settled naturally but was not observed until the third day after settlement. At this time there were 218 animals present.

Table II

Total number of animals, date of settlement and depth at which populations were reared. Note that the number of animals in population H is a re-calculated figure from 218 animals observed on third day (see text)

Population	A	B	C	D	E	F	G	H	I
Total no. of animals	80	488	173	148	72	105	79	271	34
Date of settlement	23.6.61	30.6.61	7.8.61	7.8.61	11.8.61	11.8.61	31.1.59	24.9.61	23.10.61
Depth in feet	4	4	4	7	4	7	7	7	7
Artificial or natural settlement	A	A	A	A	A	A	A	N	N

TABLE III

Survival rate (lx) in 9 populations of young Ascidia nigra, *presented as cohorts of 1000 animals each.   For further data on these populations, see Table II.*

No. of days since settlement	Population								
	A	B	C	D	E	F	G	H	I
0	1000	1000	1000	1000	1000	1000	1000	1000	1000
1	950	—	960	919	—	—	—	—	—
2	862	846	873	865	—	—	—	—	—
3	525	814	815	831	639	638	—	803	941
4	437	773	—	—	583	571	342	—	—
5	362	748	—	—	—	—	—	635	—
6	—	619	—	514	333	495	—	—	—
7	—	518	486	—	208	448	—	—	—
8	275	—	—	—	181	400	—	565	588
9	—	—	—	365	—	—	215	—	—
10	262	434	231	—	111	286	—	458	—
11	—	—	—	223	—	—	—	—	500
12	250	—	—	—	55	267	—	—	—
13	—	348	145	—	—	—	—	362	—
14	238	—	—	162	42	210	—	—	—
15	—	—	87	—	—	—	—	—	353
16	—	299	—	135	—	—	76	273	—
17	—	—	58	—	42	162	—	—	—
18	238	279	—	122	—	—	—	—	—
19	—	—	—	—	14	76	—	177	—
20	—	—	17	—	—	—	—	—	—
21	225	240	—	—	14	67	—	—	176
22	—	—	17	81	—	—	—	—	—
23	225	200	—	—	—	—	51	—	—
24	—	—	0	61	—	—	—	48	—
25	—	191	—	—	—	—	—	—	118
26	200	—	—	—	0	57	—	—	—
27	—	—		—		—	—	30	—
28	187	154		—		—	—	—	58
29	—	—		61		—	—	—	—
30	—	—		—		—	38	—	—
31	—	—		—		—		—	—
32	—	129		—		—		—	—
33	175	—		—		48		—	—
34	—	—		—		—		11	—
35	—	127		—		—		—	—
36	—	—		—		—		—	—
37	—	—		41		—		—	—
38	—	—		—		—		—	—
39	—	119		—		—		—	—
40	—			—		—		—	—
41	—			—		—		—	—
42	—			—		29		—	30
43	175			—				—	
44				—				—	
45				20				3.7	

The figure of 271 given in Table II is a re-calculated value of the probable size of the original population when it settled. This has been calculated from the mean of the known survival in populations D, F and I on the third day; these populations are chosen because they were reared at the same level as H, and data are available for the third day of life. The re-calculation introduces a possible error in the data, but without it we cannot compare this interesting natural population with those which were artificially reared. The extent of the error is not large and is confined to the position of the first point on the survival curve and the percentage of survivors remaining at the terminal point; the intervening curve would be displaced but not altered in form. The percentage of survivors at the end might be depressed as low as 0.24% or elevated to 0.45% as against 0.37% resulting from the re-calculation. These figures are based on the assumption that the highest mortality in the first three days might be equivalent to that shown by population A (47.5%), which would result in 0.24% of survivors at the terminal point, and the extreme possibility that there had been no mortality in the first three days when there would be 0.45% of survivors at the end; both alternatives are improbable.

The data are presented graphically in Figures 1 to 4. It will be apparent from these figures that there is not one clear-cut type, but several groups, of survival patterns. Populations A and B form one grouping, with 17.5% and 12% survival after 40 days; populations D, F, G and I form a second grouping, with between 2% and 3% survival after the same period, and populations C and E form a third group, all of which had been lost by the twenty-fifth day. Population H, the large, naturally settled population, followed the pattern of D, F, G and I at first but diverged markedly after about 20 days; it is discussed further below.

These different survival patterns can, to some extent, be related to differences in the environment under which the populations were reared. If we consider populations A, B, C and E at first, we find that all four populations, derived from artificial fertilizations, were reared in the sea at a depth of four feet below the surface; since they were suspended from a floating raft this depth remained constant, irrespective of tidal movements. Populations A and B were derived from fertilizations at the end of June, C and E were from fertilizations in early August. In early August and through September of 1961, when these experiments were carried out, there was a dense settlement of algal spores and subsequent growth of filamentous algae on all frames at the four-foot level. The frames with populations A, B, C and E were all equally affected by algal growth. The continued survival of populations A and B and the total loss of populations C and E under these conditions may be explained by the fact that the survivors in A and B were, by this time, quite large animals, while the survivors in C and E were still in the early stages of development and probably more susceptible to smothering by algae. We do not have sufficient data on other environmental factors at these times to be certain that it was the algae and not something else which caused the rapid decline of C and E. However, we may now compare these two populations with populations D and F, also derived from artificial fertilizations in early August. D and F were reared at 7 feet below the surface, left 3% of survivors after 6 weeks and did not have the same dense growth of algae on the frames as did C and E. This, then, strengthens the conclusion that the dense algal growth may have been responsible for the decline in populations C and E. Populations C and D were derived from the same
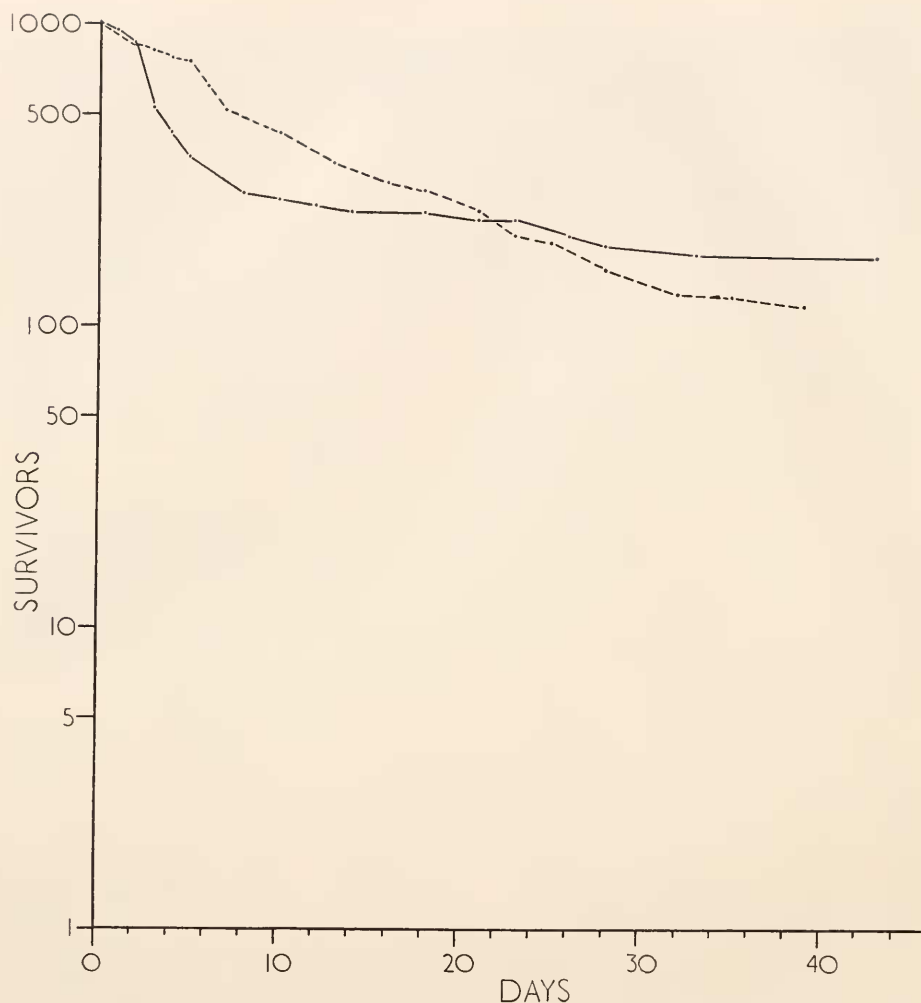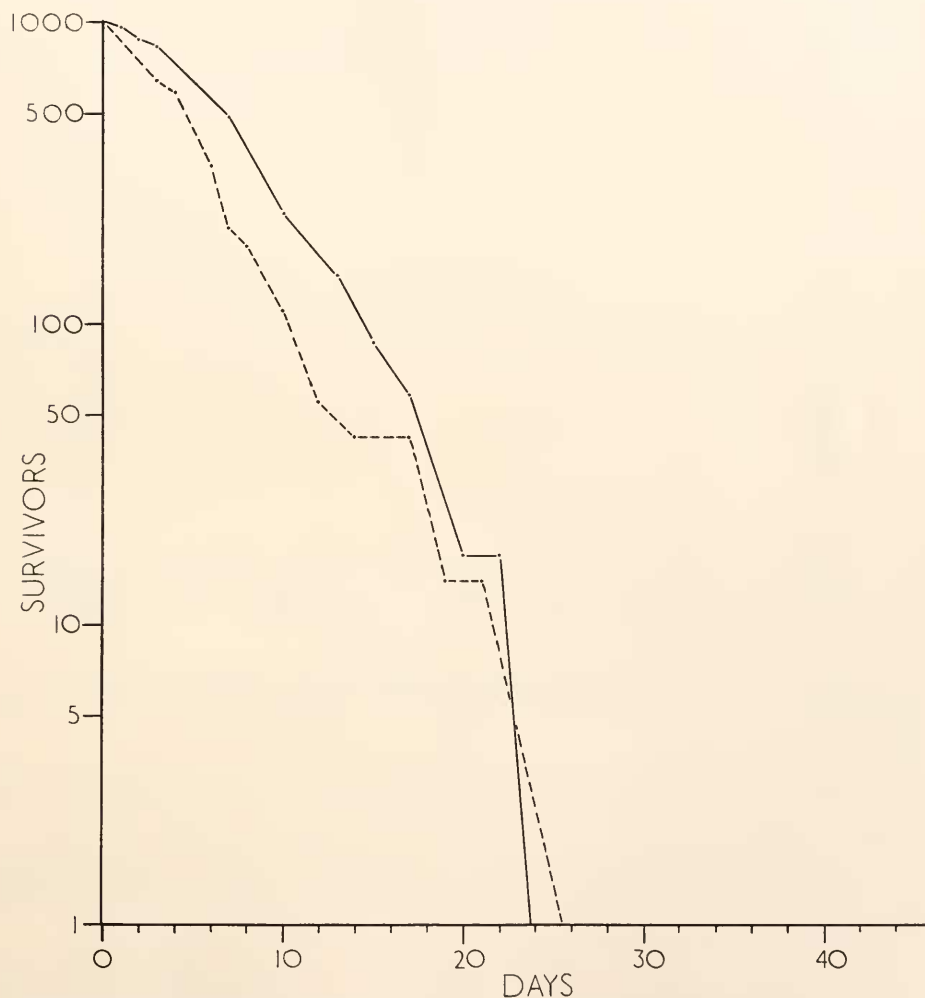
FIGURE 1. Survival in two populations of young ascidians, A (—————) and B (— — — —), settled in June, 1961, and reared four feet below the sea surface.

fertilization and were placed in the water on August 7th, the one three feet below the other in the water; the deeper one, D, with little algal growth, had a higher rate of survival than the upper algal-covered population C. Similarly, populations E and F were derived from a single fertilization on August 11th; F was kept three feet below E and had more survivors.

The high level of survival in populations A and B in comparison with D and F is also of interest but not readily explained. A and B were derived from fertilizations in late June and were maintained four feet from the surface; D and F were derived from fertilizations in early August and were maintained 7 feet from the surface. The difference in survival between the two sets of populations may be due

to an environmental factor, possibly a biotic factor concerned with competition from other sessile organisms. Similar sorts of communities developed with each population dominated by cirripedes, serpulids and colonial ascidians. The data on the associated fauna and flora are necessarily rather superficial but there is nothing in the record to suggest that one community differed much from the other. Attention should be drawn to the fact that June is one of the months when breeding in *A. nigra* is normally at a minimum (Goodbody, 1961), suggesting that this is a poor time of year for breeding and subsequent survival. August, on the other hand, is at the beginning of the autumnal rise in breeding activity of this species, suggesting that environmental conditions for breeding and survival of the young are improving.

FIGURE 2. Survival in two populations of young ascidians, C (————) and E (— — — —), settled in August, 1961, and reared four feet below the sea surface.
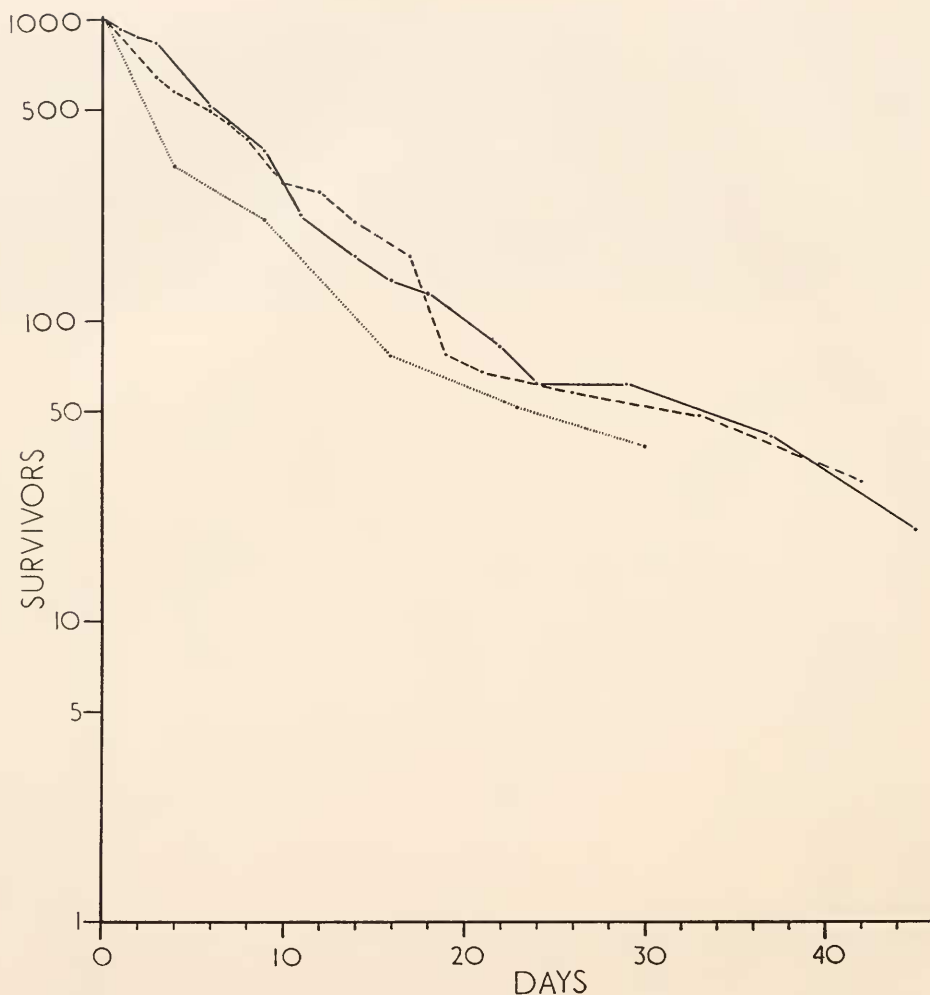
FIGURE 3. Survival in three populations of young ascidians reared 7 feet below the sea surface. D (————) and F (————) were settled in August, 1961. G (..........) was settled in January, 1959.

It is surprising, therefore, to find that the June population shows such a high level of survival.

There remain three other populations to be discussed. G was a population set up in January, 1959, and maintained 7 feet below the surface. It was followed for only 30 days and until then showed a survival pattern closely similar to those populations settled in August and maintained at 7 feet.

Finally, there are data on two populations derived from fertilizations in the sea, which settled naturally on slides at 7 feet. H was a large population settling on slides which already had two weeks' growth of barnacles and other sessile forms. I settled two days after slides had been placed in the water and is therefore

more nearly comparable to the artificial settlements; it is, however, a small population compared to any of the others.

Population I shows a survival curve closely approximating that of the artificial populations D, F and G, reared at 7 feet, and like them had 3% survival at the end of 6 weeks. However, it must be emphasized that this represents no more than one animal surviving. Population H follows the pattern of D, F, G and I until about the twentieth day, when the curve falls sharply, and the final survival at the forty-fifth day is only 0.37%, but this also represents only one surviving animal. Nevertheless, the curve merits some attention, as there is a possible explanation for the divergence between it and the others. The animals in this population settled in a
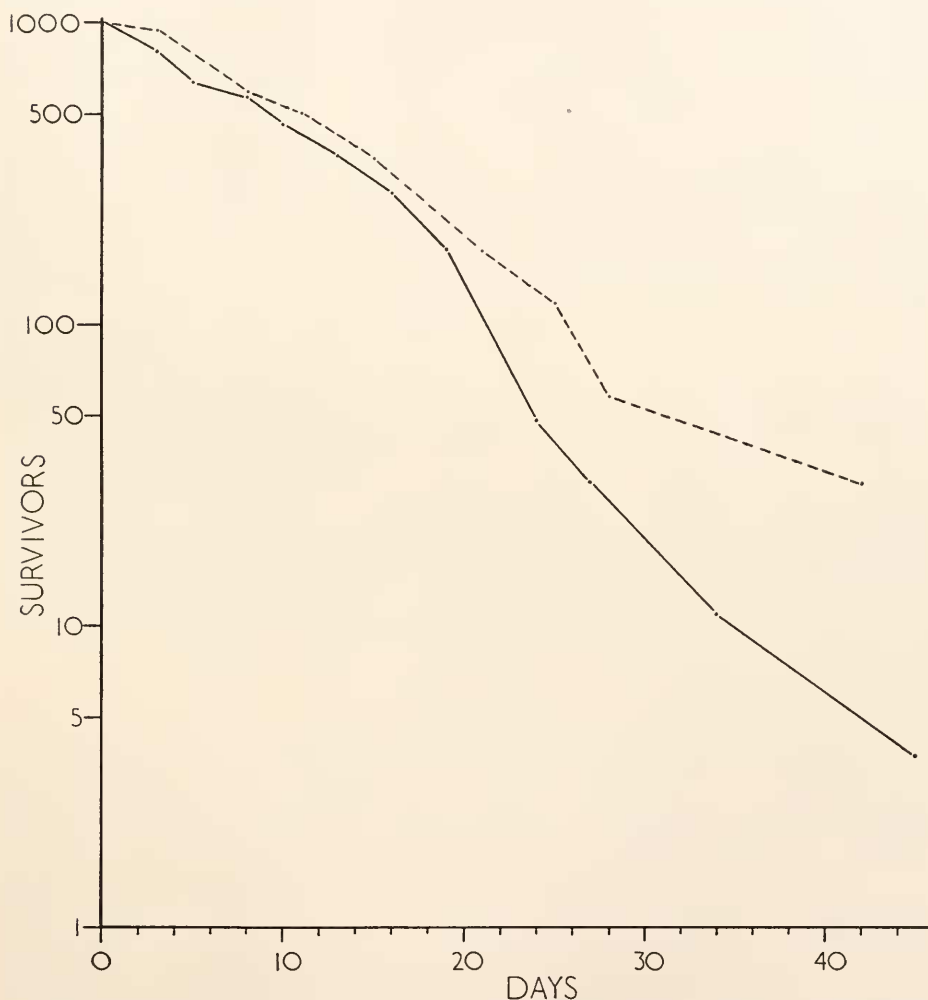
FIGURE 4. Survival in two naturally settled populations of young ascidians, 7 feet below the sea surface. H (————) settled in September, 1961, and I (— — — —) settled in October, 1961. The data for H have been re-calculated to obtain the first point on the curve (see text).

population which was already 16 days old, whereas those in population I settled in one which was two days old, and D, F and G were first colonizers. We might expect therefore that competition from organisms already in possession of the slides would cause heavy mortality in population H, but we would also expect this to occur in the youngest stages from about the second day to the nineteenth day, that is, between the onset of feeding and the appearance of a fully formed black ascidian. This, in fact, does not appear to have happened and during this phase of development the survival curve follows closely that of the populations which settled early in the life of the community. The divergence between the curves does not take place until the point at which the animals had reached the opaque black stage. We must, therefore, look for another explanation, and this may be found in the nature of the community which developed in association with this population. The usual community developing in association with these populations comprised green algae (*Enteromorpha*), cirripedes (*Balanus*), serpulid polychaetes (*Eupomatus*), colonial ascidians (*Didemnum candidum* Savigny), *Diplosoma macdonaldi* Herdman and *Symplegma viride* (Herdman), lamellibranchs (probably *Ostrea equestris* Say) and a large errant fauna of amphipods, copepods, turbellarians, polychaete annelids, herbivorous gastropods (on the algae) and occasionally nudibranchs. The community in association with population I differed markedly from all others in that a large population of pyurid ascidians (*Microcosmus exasperatus* Heller) developed and was already in evidence by the time the *Ascidia nigra* were 20 days old; they eventually became the dominant organism in this community. Pyurid ascidians in general belong to a later stage in the natural succession of inshore sessile communities in Jamaica (unpublished observations), and may in this case have played some part in displacing the *A. nigra*, either by competition for food or by squeezing them off their attachment bases.

Conclusions

The general picture emerging from these data on *Ascidia nigra* is that survival of between 2% and 3% may be normal for a population at 7 feet after the first 6 weeks of life. All the artificial populations and one of the natural populations reared at 7 feet below the sea surface exhibited this pattern of survival, but this is not true of populations reared at the four-foot level. Of four populations reared at this level, two left no survivors and two left between 12% and 18% survivors.

It is probable that the two populations which left no survivors were affected adversely by dense algal growth, but until more information is available, we can only speculate on the high survival of the other two. This extreme divergence between growth of populations reared at the four-foot level, and the smaller divergence between population H and the other populations reared at 7 feet, does, however, focus attention on the effects of environmental differences on survival rates of developing ascidians. These differences can be considered as having dimensions in both space and time. On the one hand, divergence between populations at the four-foot level is assumed to be the result of seasonal differences in the settlement of other organisms at that level. On the other hand, the difference noted between populations at different vertical levels is probably indirectly due to differences in illumination; this results in different biotic communities developing in association with, and in competition with, the ascidians. In both cases it is assumed that dif-

ferences in the associated community are responsible for the differences in survival, but the community differences are due in the first instance to seasonality and in the second to spatial separation. Similarly, it is suggested that the divergence between the two natural populations, H and I, is a biotic effect due to direct competition between two species of ascidians. These are obviously very complex problems and we hope to progress further with them in the next few years.

At the outset of this work it was thought possible that there might be some correlation between mortality rate and the stage of development of the young ascidian. There is no clear-cut evidence of such a correlation from these data, and the most that can be said is that the highest mortality occurs before the animal becomes completely black, about the nineteenth day. The causes of mortality in the young ascidian are varied. Some zooids are undoubtedly eaten by flatworms and occasionally by young polychaete worms; others have been found dead on the slide with numerous ciliate protozoa inside the empty test. Other ascidians appear to starve to death in the shadow of a barnacle; ascidians growing very close to barnacles have been observed to shrivel slowly from one inspection to another, as if they were unable to obtain sufficient food in competition with the barnacle. A further cause of mortality is due to spatial competition with colonial ascidians such as *Diplosoma macdonaldi, Didemnum candidum* and *Symplegma viride;* all these species have been seen to smother young *A. nigra* as the colony spreads across the slide. Mention has already been made of the possibility that dense mats of algal growth may be responsible for ascidian mortality, presumably by preventing an adequate flow of food from reaching the ascidian.

Summary

1. A time scale for the development of *Ascidia nigra* is given, from fertilization of the egg to the completion of black pigment formation. Embryonic and larval development are completed in less than 24 hours, and a functional ascidian is developed within 48 hours of larval settlement. Pigment first appears about the thirteenth day and the animal is completely black by the eighteenth or nineteenth day. The whole developmental process is completed about six times faster than in temperate-water ascidians at temperatures about 11° C. less.

2. The survival of populations of ascidians has been followed from settlement to the end of the sixth week of life. Most populations left only 2% to 3% survivors, but some left none and one as many as 17.5% survivors. These differences are discussed and are assumed to be due to differences in the associated biotic community arising from differences in illumination and season of growth.

3. Death is due to a number of factors, including predation by flatworms and polychaete annelids, competition for food and for space with other sessile animals or algae, and possibly in some cases protozoan infection.

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